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Fission yeast homologue of Tip41-like proteins regulates type 2A phosphatases and responses to nitrogen sources

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Abstract

A fission yeast (*Schizosaccharomyces pombe*) gene encoding a member of the TIP41-like protein family was identified and characterized. Deletion of the fission yeast *tip41* gene leads to slower growth when ammonium chloride is the nitrogen source, but the growth rate is not affected when adenine is the nitrogen source. The *tip41* mutant cells also enter the G1 phase of the cell cycle earlier than wild-type cells in response to nitrogen starvation. Overexpression of $tip41^{+}$ causes cell death, spherical cell morphology and blocks the shift to G1 phase upon nitrogen starvation. Overexpression of $tip41^{+}$ increases the activity of type 2A phosphatase. In a *ppa2* deletion strain with reduced PP2A activity, overexpression of $tip41^{+}$ no longer blocks the shift to G1 upon nitrogen starvation. These results suggest that fission yeast Tip41 plays a role in cellular responses to nitrogen nutrient conditions at least partly through regulation of type 2A phosphatase activity.

Keywords: *Schizosaccharomyces pombe*; Tip41-like family protein; Nitrogen starvation; PP2A

1. Introduction

The TOR pathway is a major regulatory pathway controlling cell growth in response to nutrients in eukaryotes ranging from yeast to mammals (reviewed in [1–3]). For example, in the budding yeast *S. cerevisiae*, growth on low quality nitrogen sources such as proline inactivates TOR ultimately leading to upregulation of genes required to use these secondary nitrogen sources [2,3]. Type 2A and related phosphatases are major downstream effectors of TOR regulation during this response to nitrogen source in budding yeast [4,5]. On good nitrogen sources such as ammonium or glutamine, TOR is activated and represses SIT4, a phosphatase related to type 2A protein phosphatases. Upon inactivation of TOR during growth on a poor nitrogen source, SIT4 is activated to dephosphorylate and activate the GLN3 transcription factor which increases the transcription of genes required to use poor quality nitrogen sources [6–8]. TOR regulates SIT4 through the TAP42 protein which binds to and inhibits the catalytic subunit of SIT4 [4,5,9]. Inactivation of TOR by growth on a poor nitrogen source leads to dephosphorylation of TAP42 and dissociation from SIT4 resulting in activation of SIT4.

TIP41 is a recently described regulator of TAP42 required for efficient TOR regulation of SIT4 [10]. TIP41 binds to TAP42 and inactivates it by preventing its interaction with SIT4. Active TOR phosphorylates TIP41 which prevents its binding to and inactivation of TAP42 whereas inactive TOR leads to dephosphorylated TIP41 which binds to and inactivates TAP42. The end result of this regulatory cascade is that inactivation of TOR by growth on a poor nitrogen source leads to activation of the type 2A related phosphatase SIT4 through the intermediary regulatory proteins TAP42 and TIP41 [2,10].

A fission yeast hypothetic protein was identified during screenings in fission yeast (*Schizosaccharomyces pombe*) for multicopy suppressors of Vpr (accession number:

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SPCC4B3.16), a HIV-1 viral protein that has been shown to induce cell cycle G2/M arrest through PP2A [11–13]. This fission yeast hypothetic protein was designated as Tip41 based its significant sequence homology to the TIP41-like proteins. It is intriguing that both Vpr and Tip41 potentially regulate PP2A and this regulation may be related to the suppression of Vpr activity by Tip41. Since Tip41 is a novel fission yeast protein, the goal of this study was to characterize its biologic properties and potential relationship with PP2A. The potential effect of fission yeast Tip41 on the Vpr activities will be described elsewhere.

Budding yeast TIP41 is the only member of this family which has been shown to act as a regulator of type 2 and related protein phosphatases [10], and no other TIP41-like protein has thus far been shown to have these roles. Identification of the TIP41-like protein in *S. pombe* thus enabled us to test the potential role of the fission yeast TIP41-like protein in cellular responses to nitrogen nutrient conditions and to determine if Tip41 regulates PP2A.

2. Materials and methods

2.1. Yeast strains and media

Genotypes and sources of *S. pombe* strains and plasmids used in this study are summarized in Table 1. The *S. pombe* parental wild type (Q812) and mutant *tip41*::*hisG* cells containing various plasmid constructs were maintained in standard Edinburgh minimal medium (EMM) [14] supplemented with uracil and adenine at 75 μg/ml. To avoid problems from the *leu1-32* allele which requires high concentrations of leucine [15], strains were usually transformed with the leu-selectable plasmid pYZ1N [16] before they were used in experiments. Thiamine was added to the medium at a final concentration of 20 μM to repress expression from the regulated *nmt1* (no message in thiamine) promoter [17]. The EMM plates contain 93 mM NH_4Cl, and plates referred to as −NH4Cl are EMM media without NH4Cl. *S. pombe* can use adenine as a sole source of nitrogen [18], and the cells use the 0.44 mM adenine in the −NH4Cl plates as a nitrogen source.

2.2. Molecular cloning and nucleotide sequence determination of the *S. pombe tip41*$^+$ gene

The *tip41*$^+$ cDNA was originally isolated from a cDNA library (a gift from Greg Hannon) made from *S. pombe* wild type strain SP972 in pREP3X [19] by PCR amplification of the *tip41*$^+$ coding sequence and inserting it into the pYZ1N plasmid [16]. The complete nucleotide sequence of the *tip41*$^+$ cDNA insert in pYZ1N was determined with the ABI automated DNA Sequencer Model 377 (Perkin-Elmer, CA).

Table 1
Schizosaccharomyces pombe strains and plasmids

Names	Genotype	Sources
Strains		
Q812	wild type, *h*$^-$ *leu1-32, ura4-D18*	[35]
Q813	wild type, *h*$^+$ *leu1-32 ura4-D18*	[35]
SP223	wild type, *h*$^-$ *ade6-216 leu1-32, ura4-294*	Laboratory collection
ZB24int	A single copy integrant of *tip41* in SP223	This study
Δ*tip41*	*h*$^-$, *tip41*::*hisG leu1-32 ura4-D18* isogenic to Q812	This study
Δ*ppa2*	*h*$^-$, *leu1 ura4 ppa2*::*ura4+*	[32]
Plasmids		
pYZ1N	Derivative of pREP1N, *leu1* selection	[16]
pYZ1N-*tip41*	*tip41* in pYZ1N	This study

2.3. Construction of the *tip41*::*hisG* deletion/disruption mutant

The *tip41*$^+$ ORF was replaced by *hisG-ura4*$^+$*-hisG* fragment to generate a *tip41*::*hisG-ura4*$^+$*-hisG* disruption mutant using a one-step gene procedure described previously [20,21]. Briefly, the *hisG-ura4*$^+$*-hisG* fragment was PCR amplified using primers containing 70 bases of sequence immediately adjacent to the *tip41*$^+$ ORF to generate an *hisG-ura4*$^+$*-hisG* fragment flanked at each end with 70 base pairs of *tip41*$^+$ sequence. The PCR product was purified with a Qiagen column (Qiagen, CA) and used to transform the *S. pombe* Q812 strain in which the *ura4* gene has been deleted *(tip41*$^+$ *, h - , leu1-32, ura4-D18*). The *ura+* transformants were screened by PCR for colonies in which the *tip41* ORF had been deleted, and the *tip41*::*hisG-ura4*$^+$*-hisG* disruption was confirmed by DNA sequencing of the *tip41-hisG* junctions. To generate a *ura4*$^-$ strain isogenic to the parental strain Q812, Ura$^-$ cells were selected on 5-fluoro-orotic acid. These Ura$^-$ colonies result from homologous recombination across the *hisG* repeats to delete the *ura4*$^+$ gene to generate a strain in which the *tip41*$^+$ ORF has been replaced by the *hisG* fragment [21]. The presence of the *hisG* fragment at the *tip41*$^+$ locus was confirmed by PCR.

2.4. Construction of a *S. pombe* strain that can stably express *tip41*$^+$ upon gene induction

The *tip41*$^+$ gene, cloned into the pRIP4 vector [17], was integrated into the *S. pombe* chromosome of strain SP223 at the *ura4* locus using a standard procedure [22]. Single copy integration of the *tip41*$^+$ gene was confirmed by PCR and DNA sequencing analyses. The resulting strain is named ZB24int.

2.5. Inducible overexpression of *tip41*$^+$ gene

Fission yeast cells carrying *leu1*-selectable plasmids were maintained on agar plates of standard Edinburgh minimal medium (EMM) supplemented with adenine and uracil at 75 μg/ml and thiamine added at 20 μM to repress *tip41*$^+$ expression from the *nmt1* promoter as described previously [13,16,17]. Cells in liquid media were grown at 30 °C with constant shaking at 200 rpm unless noted otherwise. For *tip41*$^+$ induction in liquid medium, cells containing the *tip41*$^+$ plasmid were first grown to stationary phase in the presence of 20 μM thiamine. Cells were then washed three times with distilled water, diluted to a final concentration of approximately 2×10^5 cells/ml in 10 ml of the appropriately supplemented EMM medium with or without thiamine.

2.6. Measurement of cell cycle profile by flow cytometry

The procedure used to prepare *S. pombe* cells for flow cytometry analyses was modified from [23]. Briefly, cells containing pYZ1N-*tip41*$^+$ were grown to stationary phase in 5 ml of EMM medium containing thiamine with constant shaking at 30 °C. A 1 ml aliquot of culture was collected, washed three times with distilled water to remove thiamine, and re-inoculated into 5 ml low nitrogen media (LN) containing 2.5 mM NH4Cl at a concentration of 5×10^5 cells/ml with or without thiamine. Cells were collected at various time intervals and fixed with 7 ml of 95% ethanol. Before flow cytometry analysis, cells were treated with RNase A (5 μg/ml) in 50 mM sodium citrate (pH 7.0) and stained with propidium iodine (Sigma, P-4170). The level of fluorescence in individual cells was analyzed on a FACSCAN (Becton Dickinson) using CellQuest software (Becton Dickinson). Ten thousand events were collected and the level of cells in G1 and G2 was determined as the FL2 fluorescence parameter.

2.7. Fluorescence microscopy

A Leica fluorescence microscope equipped with a high performance CCD camera (Sensicam) and OpenLab software (Leica Inc.) was used for all imaging analyses. For the TRITC-phalloidin staining of actin, cells were collected onto Whatman 934-AH glass microfiber filters and fixed with 100% ice-cold

methanol at −20 °C for 20 min. The protocol used for TRITC-phalloidin staining is described in [24].

2.8. Type 2A protein phosphatase assay

Cell extracts were prepared by treatment of cells with 1 mg/ml Novozyme 234 followed by sonication and centrifugation in PP2A storage buffer (25 mM Tris-EDTA, pH 7.5, 1 mM β-mercaptoethanol, 2 mM EDTA, 0.1 mM PMSF, 0.1% triton X-100) containing protease inhibitor cocktail (1 tablet/10 ml complete mini, Roche). Activity in the extracts was determined with the Ser/Thr Phosphatase Assay System following the manufacturer's directions (Promega Corp, Madison, WI). Briefly, the free phosphate was removed from the extracts by fractionation on Sephadex G-25, and 35 μg protein were used in the assay. The phosphopeptide RRA(pT)VA used in the assay is a good substrate for type 2A, 2B and 2C protein phosphatases. The assay is made specific for PP2A by having EGTA and no Mg in the assay buffer. The EGTA chelates the calcium necessary for PP2B activity and the absence of Mg prevents PP2C activity. The free phosphate generated by PP2A activity on the phosphopeptide substrate is measured on a microplate reader after adding a molybdate dye.

3. Results

3.1. Identification and molecular cloning of the tip41$^+$ gene

HIV-1 viral protein R (Vpr) affects multiple basic cellular activities both in human and fission yeast cells (for reviews, see [25,26]. One of these Vpr effects is the induction of cell cycle G2/M arrest through protein phosphatase type 2A (PP2A) [11–13]. In screening for multicopy suppressors of HIV-1 Vpr, we identified a cDNA clone that specifically suppressed Vpr-induced cell elongation, a classic sign of cell cycle G2 arrest ([11];unpublished data). Since this fission yeast gene encodes a hypothetical protein not previously characterized, this newly identified fission yeast gene was named *tip41* based on its significant protein sequence homology to the TIP41-like proteins (GenBank accession number: SPCC4B3.16).

Consistent with the nucleotide sequence found in the *S. pombe* database (http://www.genedb.org/genedb/pombe/index.jsp), the *tip41*$^+$ gene has no introns and encodes a protein containing 252 amino acids, with a predicted molecular weight of approximately 29.4 kDa. Sequence comparisons indicate that the *S. pombe* Tip41 protein belongs to the TIP41-like protein family with the homology spanning aa41 to 218. Comparison of the *S. pombe* Tip41 sequence to five other TIP41-like sequences from budding yeast, fly, nematode, *Arabidopsis*, and human indicated a high degree of similarity (29 to 34% identical; 49–55% similar). Phylogenetic analysis further confirmed that these proteins are evolutionarily related to each other, with *S. pombe* Tip41 being most closely related to budding yeast TIP41.

3.2. A Δtip41 strain is viable but has a cold sensitive growth phenotype with ammonium choride as a nitrogen source

A *tip41*::*hisG* deletion mutant was created in *S. pombe* by replacing the *tip41*$^+$ ORF with *hisG* using a PCR-based method [20,27] combined with the *hisG-ura4*$^+$*-hisG* disruption cassette [21]. The *tip41*::*hisG* mutant (Δ*tip41*) strain is viable at 30 °C and 25 °C with no obvious differences in cell morphology (Fig. 1A). However, the *tip41*::*hisG* deletion mutant grows slower than the wild-type strain. Growth of the Δ*tip41* strain on plates after 6 days incubation at 30 °C is about 10-fold slower than wild type (Fig. 1B-a, left panel). In liquid media at 30 °C, the Δ*tip41* strain has a doubling time of 5.4 h compared to 4.7 h for wild type.

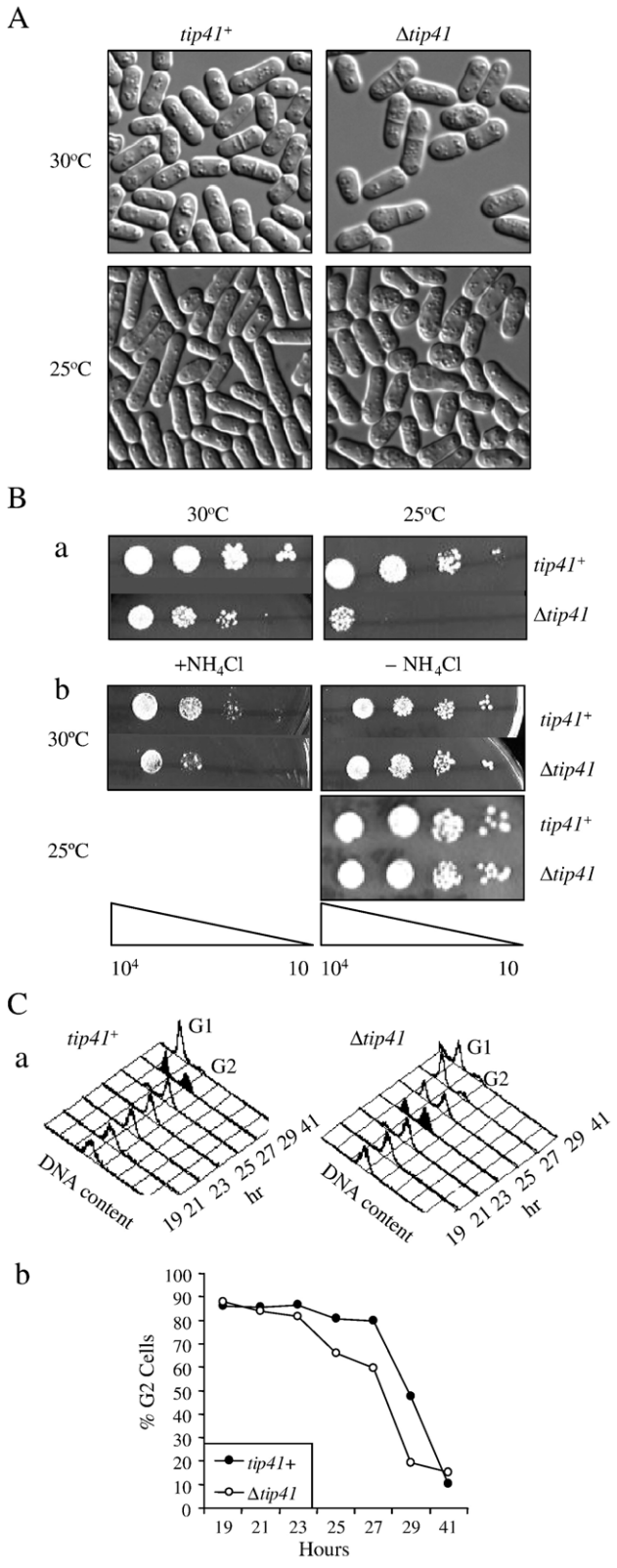
The Δ*tip41* mutant strain shows even slower growth relative to wild type at 25 °C (Fig. 1B-a, right panel). For growth on plates at 25 °C, the Δ*tip41* mutant showed about a 10^3 -fold decrease in growth compared to wild-type cells (Fig. 1B-a, right panel). There is also slow growth of the Δ*tip41* mutant cells in liquid media at 25 °C with an average doubling time of 15.8 h compared to 7.1 h for wild type cells. It should be noted that both yeast cell growth and viability affect the results of the spot dilution assay. At the lower temperature (25 °C), lack of colony formation at lower dilutions might indicate cell death due to *tip41* deletion. However, the formation of small colonies in the highest concentration spot indicate at least some level of cell survival under this condition. Together, these observations indicate that *tip41*$^+$ is a nonessential gene under normal growth conditions but it is cold sensitive and required for optimal cellular growth and possibly survival when ammonium chloride is the nitrogen source especially at lower temperature.

3.3. Requirement for Tip41 depends on the nitrogen source

Given that Tor1 plays a role in responding to nitrogen conditions in fission yeast [28] and that Tip41 may regulate the Tor1 pathway based on the analysis of TIP41 in budding yeast [10], the possible role of Tip41 in responding to nitrogen conditions was evaluated in two ways. First, growth of wild type and Δ*tip41* was compared on plates with no ammonium chloride. On these plates, yeast cells are able to grow using adenine as the sole nitrogen source [18]. In contrast to the results above on plates containing ammonium chloride where the Δ*tip41* strain grew slower than the wild-type strain (Fig. 1B upper panels and lower left panel), there was no significant difference in growth between the wild-type and Δ*tip41* strains at 25° or 30 °C on plates with no ammonium chloride (Fig. 1B, lower middle and right panels).

The second way that a possible connection between nitrogen conditions and Δ*tip41* was examined is the cell cycle response to nitrogen starvation. When wild type cells are growing with sufficient nutrients, the cells are mostly in the G2 phase of the cell cycle when assayed by flow cytometry. However, when wild-type cells are starved for nitrogen, they enter stationary phase arrested in the G1 phase [23]. The wild-type and Δ*tip41* strains were compared under conditions of nitrogen starvation by inoculating low nitrogen media which contains 2.5 mM ammonium chloride. Under these conditions, growth continues until the nitrogen sources are depleted and the cells arrest in the G1 phase of the cell cycle as they enter stationary phase [23]. In these experiments, the wild type cells begin to shift from G2 to G1 phase by 29 h after inoculation of low nitrogen media, whereas

Δ*tip41* mutant cells begin to enter the G1 phase by 25 h (Fig. 1C). Even though 4 h represents a small change, i.e., approx. one doubling time of fission yeast under this growth condition, this difference between the wild type and Δ*tip41* deletion mutant is consistent and reproducible.



3.4. Overexpression of *tip41*$^+$ causes cell death, affects cell morphology and prevents accumulation of G1 cells after nitrogen starvation

The *tip41*$^+$ gene was overexpressed from the *nmt1* promoter which is strongly induced when there is no thiamine in the media [17]. When assayed by spotting cells on a plate, a 1,000 fold reduction in growth was seen when *tip41*$^+$ was overexpressed (Fig. 2A, left panel). When *tip41*$^+$ is overexpressed in liquid media, cell growth and viability are also affected. With repression of *tip41*$^+$ by thiamine in the media, the culture grows up to stationary phase at the normal rate, but when *tip41*$^+$ is expressed at a high level, growth of the culture essentially stops about 20 h after the switch to inducing media with a doubling time of greater than 20 h (data not shown). Since high level expression from the *nmt1* promoter does not reach a maximum until 12 to 14 h after the switch to inducing media [13,17], high level expression of *tip41*$^+$ stops growth within a few hours. To determine if cells remain viable after growth inhibition, cells from the induced and repressed cultures were plated on repressing media to determine colony forming units. For the repressed culture, viability remained high with more than 50% plating efficiency for all time points (Fig. 2A, right panel). In contrast, when *tip41*$^+$ was expressed at high levels, the plating efficiency rapidly decreased to less than 1% beginning by 16 h. These results indicate that overexpression of *tip41*$^+$ leads to cell death within a few hours.

Overexpression of *tip41* causes cells to become spherical and grossly enlarged at approximately 24 h after gene induction (Fig. 2B, top right). Since *S. pombe* cells normally grow uni- or bi-directionally, these morphological changes suggest that cellular growth polarity is altered. To examine this possibility, we used phalloidin staining to observe the F-actin patterns, an indication of cell growth polarity in *S. pombe* cells [29]. In *tip41*-repressed cells, normal growth and F-actin staining patterns [30,31] were found for the various stages of the cell cycle (Fig. 2B, bottom left). Phalloidin staining of actin patches at the tips of the cells, an indication of apical growth, showed that cells with monopolar or bipolar growth were present (Fig.

Fig. 1. Tip41 is nonessential but is required for optimal growth under normal nutrient conditions. (A) The *tip41*::*hisG* mutant cells are viable and show similar morphology to wild-type cells (stain Q812) at 25 °C and 30 °C when grown in liquid EMM supplemented with ade and ura. Both the Δ*tip41* and the wild-type strain carry the leu-selectable plasmid pYZ1N [16]. (B) The Δ*tip41* mutant shows reduced growth compared to wild type at 30 °C and growth relative to wild type is inhibited even more at 25 °C. Growth was determined by plating serial 10-fold dilution of cells from 10^5 to 10^2 cells per spot on supplemented EMM and incubating for 6 days (a). In the lower panels, serial dilutions of 10 to 10^4 cells were plated on EMM supplemented with ade and ura and with (b) or without ammonium chloride (right two panels). On plates without ammonium chloride, Δ*tip41* grows as well as wild type both at 25° and 30 °C. (C) The shift to G1 of the cell cycle occurs earlier in the Δ*tip41* strain. Cultures were grown in supplemented EMM with 2.5 mM ammonium chloride. Under these conditions, the cultures typically grow until they become starved for nitrogen and then enter stationary phase arrested in the G1 phase of the cell cycle [23]. The stage of the cell cycle was monitored by flow cytometry after staining with propidium iodide for wild type (a, left) and Δ*tip41* (a, right). The quantitation of the flow analyses is presented in b.

2B, bottom left). In contrast to these normal patterns of actin localization when *tip41*$^+$ was repressed (Fig. 2B, bottom a to e), cells in which *tip41*$^+$ gene expression was induced for 24 h (Fig. 2B, bottom right) did not have any obvious actin patches at the tips of the cells, and instead actin was dispersed throughout the cells. The absence of actin patches at the tips of the cells indicates that the normal growth polarity of these cells has been disrupted.

Since deletion of *tip41* caused the shift to G1 in response to nitrogen starvation to occur earlier (Fig. 1C), the effect of *tip41*$^+$ overexpression on the response to nitrogen starvation was tested. In low nitrogen medium, *tip41*$^+$ overexpression does not have as strong a toxic effect as in regular EMM growth medium (data not shown) so flow cytometric analysis is possible. It thus allows us to carry out flow cytometric analysis. In control cells in which *tip41*$^+$ gene was suppressed (Fig. 2C, left panel), the cell cycle profile shows the typical shift from predominantly G2 to the G1 phase after prolonged incubation in low nitrogen media results in nitrogen starvation [23]. About 90% of the cells were in the G2 phase for the *tip41*$^+$-Off control during the first 28 h (Fig. 2C-a, left panel; Fig. 2C-b), but by 44 h, only about 20% of the cells remained in the G2 phase indicating the expected G2 to G1 shift. In contrast, about 65–80% of the cells in the *tip41*$^+$-expressing culture remained in G2 even after prolonged incubation in low nitrogen media (Fig. 2C-a, right panel; Fig. 2C-b). Because these experiments used medium which selected for the plasmid, the G1 cells are not cells which have lost the plasmid and are starving for leucine. Since the copy number of a plasmid in cells typically displays a Poisson distribution, the small percentage of cells which are in G1 might represent those cells with few copies of the *tip41*$^+$ plasmid.

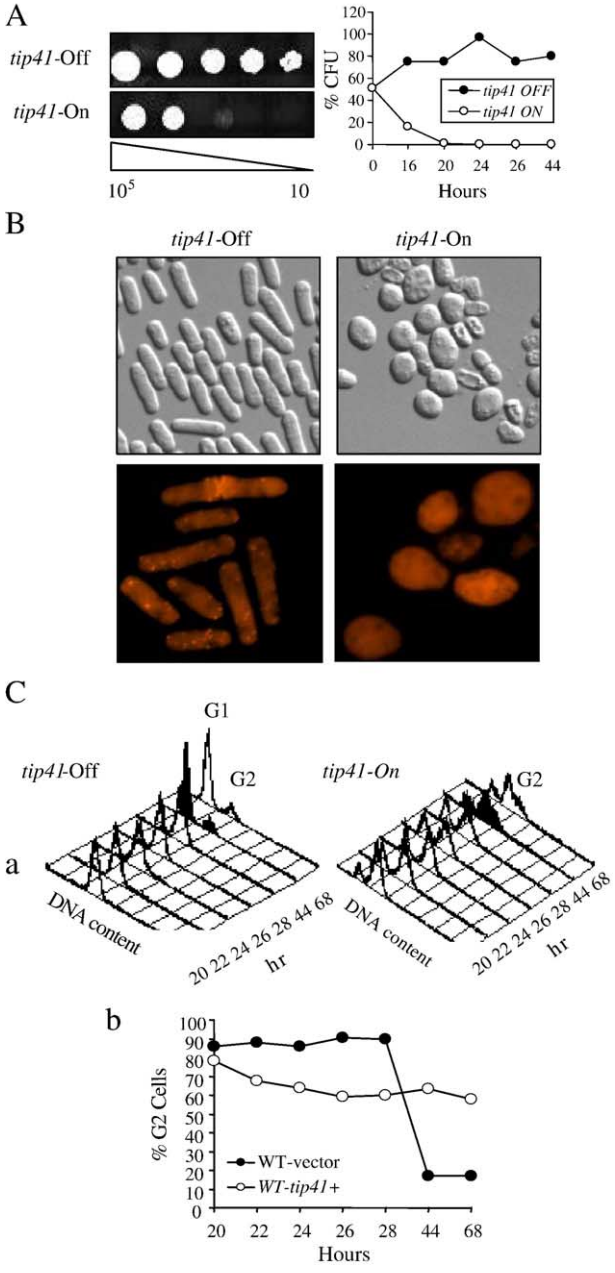


Fig. 2. Over-expression of *tip41*$^+$ causes cell death, changes cell morphology and prevents the shift to G1 after nitrogen starvation. (A) Overexpression of *tip41*$^+$ inhibits growth. Serial dilution from 10 to 10^5 cells of SP223 transformed with *tip4*1-pYZ1N were plated on media with thiamine, which represses the *nmt1* promoter, in the upper panel (*tip41*-Off), or on media without thiamine, which induces the promoter, in the bottom panel (*tip41*-On). Growth is inhibited about a 1000-fold by overexpression of *tip41*$^+$. In the right panel, cultures were incubated for the indicated times under repressing (*tip41*-Off) or inducing *(tip41*-On) conditions, and cells plated under repressing conditions to determine cell viability. Overexpression of *tip41* causes a rapid loss of viability. (B) Overexpression of *tip41*$^+$ alters cell morphology by interrupting cellular growth polarity. Under repressing conditions, cell morphology is normal (left, upper panel), but spherical, enlarged cells are seen when *tip41*$^+$ is overexpressed (right, upper panel). In the lower panels, F-actin was detected by TRITC-phalloidin staining. Left panel is under gene-repressing condition and illustrates the normal patterns of actin localization. Discrete patches are clearly seen near end of cells indicating growth polarity; right panel shows the abnormal, diffuse actin staining seen when *tip41*$^+$ is overexpressed. Micrographs were taken 24 h after *tip41*$^+$ gene induction. (C) Overexpression of *tip41*$^+$ prevents the shift to G1 upon nitrogen starvation. (a) Cultures of the vector control with pYZ1N (*tip41*-Off) and overexpression with *tip4*1-pYZ1N (*tip41*-On) were grown under inducing conditions with EMM containing 2.5 mM ammonium chloride. At the indicated times, samples from the cultures were analyzed for DNA content by flow cytometry after staining with propidium iodide. When cells go into stationary phase due to nitrogen starvation, the vector control shows the expected shift to G1 cells, but overexpression of *tip41*$^+$ inhibits this shift. The bottom panel on the right shows the quantitation of the flow cytometry (b).

3.5. Overexpression of tip41$^+$ increases PP2A activity

Fission yeast Tip41 was originally identified as a suppressor of a viral protein that activates PP2A [11–13]. In *S. cerevisia*e, TIP41, the homologue of Tip41, activates SIT4, a PP2A-like phosphatase, by interrupting the interaction of the negative regulator TAP42 with the catalytic subunit of SIT4 [10]. These two connections to PP2A suggest that Tip41 might affect the activity of PP2A. To test this possibility, we measured phosphatase activity with an assay which is selective for PP2A-type phosphatase activity (Promega Corp, Madison, WI). Extracts made from cultures with induced expression from a single integrated copy of *tip41*$^+$ showed a large increase of seven fold compared to extracts from cultures of wild-type cells ($P<0.001$, Fig. 3A). This increase was seen when the

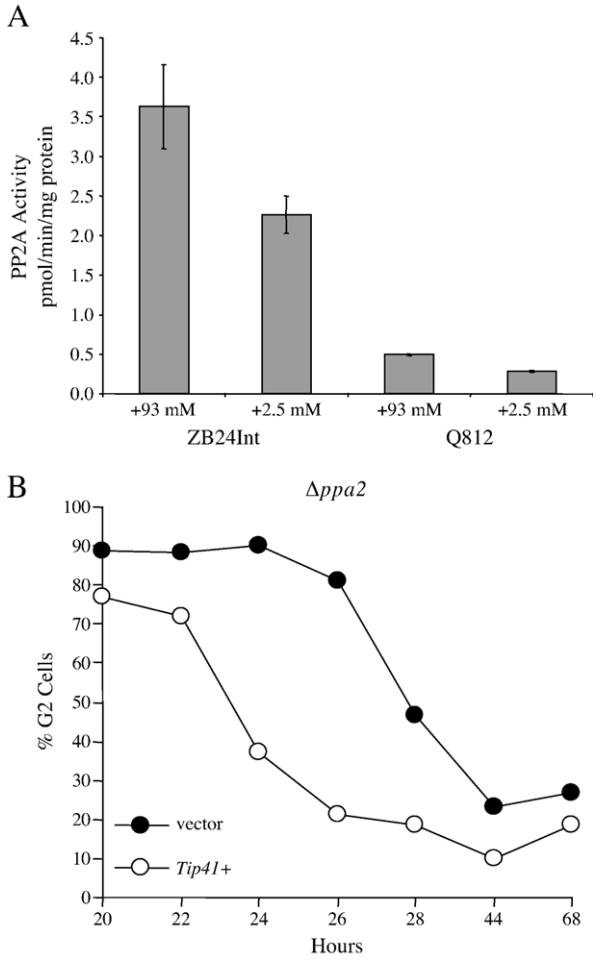


Fig. 3. PP2A plays a role in the effects of *tip41*$^+$ overexpression. (A) Overexpression of *tip41*$^+$ increases PP2A activity. The strain ZB24Int with a single integrated copy of *tip41*$^+$ expressed from the *nmt1* promoter was grown in supplemented EMM without thiamine and with either 93 mM or 2.5 mM ammonium chloride. The wild-type strain is Q812 grown under similar conditions. Extracts were made after 24 h and the amount of phosphatase activity determined using an assay specific for PP2A type activity (Promega Corp, Madison, WI). The phosphatase activity increases about seven fold when *tip41*$^+$ is overexpressed. The error bars represent the standard deviation for triplicate measurements done on three independent cultures. The differences in the phosphatase activity between cells overexpressing and not overexpressing *tip41*$^+$ is significant at the $P<0.001$ level. The differences in phosphatase activity between cells grown in 93 mM and 2.5 mM ammonium chloride are significant at the $P=0.02$ level. (B) The *.ppa2* mutation restores the G2 to G1 shift suppressed by overexpression of *tip41*$^+$. The *.ppa2* strain was transformed with *tip41*$^+$-pYZ1N or pYZ1N, and the transformants were grown in supplemented EMM media without thiamine to induce expression and 2.5 mM ammonium chloride. Samples were taken at the indicated time points and analyzed for DNA content by flow cytometry after staining with propidium iodide. Both strains show the transition to G1 as they enter stationary phase due to nitrogen starvation.

cultures were grown with high or low concentrations of ammonium chloride although growth with the low concentration resulted in a 40% decrease in phosphatase activity compared to growth on high concentrations (Fig. 3A).

This increase in PP2A phosphatase activity may play a role in the inhibition by *tip41*$^+$ overexpression of the G2 to G1 transition after nitrogen starvation (Fig. 2C). Deletion of the gene for the major catalytic subunit of PP2A, *ppa2*, reduces the PP2A activity in the cell [32,33]. When *tip41*$^+$ is overexpressed in a Δ*ppa2* strain, the transition from G2 to G1 is no longer inhibited and most of the cells arrest in G1 (compare Fig. 3B to Fig. 2C). The result with the Δ*ppa2* strain suggests that the increase in PP2A activity after *tip41*$^+$ overexpression (Fig. 3A) plays a major role in inhibiting the G2 to G1 transition after nitrogen starvation.

4. Discussion

We show here that fission yeast *tip41*$^+$ has roles in responses to nitrogen source and in regulating PP2A. The large increase in PP2A activity when *tip41*$^+$ is overexpressed suggests that Tip41 is a positive regulator of PP2A-type phosphatases. The suppression effect of *tip41*$^+$ overexpression on blocking the shift to G1 upon nitrogen starvation by a deletion of *ppa2* (Fig. 3B) suggests that this effect of *tip41*$^+$ overexpression is due to increased PP2A activity. Other effects of *tip41*$^+$ overexpression, such as growth inhibition and cell death (Fig. 2A), are not suppressed by the *ppa2* deletion (data not shown). Furthermore, overexpression of *ppa2*$^+$ does not enhance the cell death caused by *tip41*$^+$ overexpression (data not shown) indicating that increased PP2A activity is not likely to be involved in this effect of *tip41* overexpression. Detailed relationships between increased PP2A activity and the effects of *tip41*$^+$ overexpression remain to be established.

There are three lines of evidence which support the idea that the function of wild-type *tip41* is to optimize cellular metabolism for growth on media with a high concentration of ammonium chloride as opposed to low concentrations of ammonium chloride or growth on a poor nitrogen source such as adenine. First, the Δ*tip41* strain grows slower than the wild-type strain on media with high concentration of ammonium chloride (Fig. 1B-a), but there is no difference in growth rate when adenine is the nitrogen source (Fig. 1B-b). Second, the shift to G1 when cells are starved for nitrogen occurs a few h earlier in the Δ*tip41* strain compared to wild type (Fig. 1C). If deletion of *tip41* changes cellular metabolism to a state appropriate for poor nitrogen sources, an earlier shift to G1 in response to nitrogen starvation might be expected. Third, overexpression of *tip41*$^+$ reduces the shift to G1 in response to nitrogen starvation. If a function of *tip41*$^+$ is to establish metabolic conditions appropriate for growth on high concentrations of ammonium chloride, overexpression of *tip41*$^+$ might lock the cell into these conditions and prevent establishing the metabolic conditions appropriate for nitrogen starvation and thereby preventing the shift to G1.

The homology of *S. pombe tip41*$^+$ to *S. cerevisiae TIP41*, which negatively regulates the TOR pathway in *S. cerevisiae* [10], originally suggested roles for *tip41*$^+$ in nitrogen metabolism. In fission yeast, the Tor1 protein is required for the normal response to nitrogen starvation [28,34] and may be activated by nitrogen starvation. Given this evidence that a *TIP41* deletion activates the TOR pathway in budding yeast [10] and that Tor1 activation may be necessary for the appropriate response to nitrogen starvation in fission yeast

[28,34], deletion of *tip41* in fission yeast might then be expected to activate the TOR pathway and change cell physiology to a state appropriate for nitrogen limitation, a state which might be less than optimal for growth on high concentrations of ammonium chloride. A specific example of how this might be the case comes from budding yeast where the Tor pathway in budding yeast strongly regulates an ammonium ion permease [7,8]. If deletion of *tip41*, for example, downregulates ammonium ion permease, the deletion strain might show reduced growth on high concentrations of ammonium chloride but have no effect on growth with adenine as a nitrogen source. Obviously, these possibilities need to be further tested.

The sequence homology of Tip41 to budding yeast TIF41 also supports the idea that *tip41*$^{+}$ regulates PP2A since TIF41 has been shown to regulate the activity of the PP2A-type phosphatase SIT4 in budding yeast [2,10]. TIP41 is thought to activate SIT4 by binding to the negative regulator TAP42 which in turn binds to the catalytic subunit of the phosphatase [2,10]. Fission yeast has a homologue of TAP42, the SPCC63.05 gene with 25% identity and 46% similarity, but it has not yet been functionally characterized and it is unknown whether this homologue interacts with Tip41 and PP2A-like phosphatases. TIP41 is the only member of this protein family whose functional roles have been characterized in detail and been shown to be part of the TOR regulatory pathway [2,10]. The identification of *tip41*$^{+}$ as a member of the TIP41 family provides a second simple genetic organism to define and compare the roles of the highly conserved TIP41 gene family in the TOR pathway, the regulation of type 2A phosphatases and cellular activities.

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References

[1] N. Hay, N. Sonenberg, Upstream and downstream of mTOR, Genes Dev. 18 (2004) 1926–1945.
[2] E. Jacinto, M.N. Hall, Tor signalling in bugs, brain and brawn, Nat. Rev., Mol. Cell Biol. 4 (2003) 117–126.
[3] T. Schmelzle, M.N. Hall, TOR, a central controller of cell growth, Cell 103 (2000) 253–262.
[4] C.J. Di Como, K.T. Arndt, Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases, Genes Dev. 10 (1996) 1904–1916.
[5] K. Duvel, J.R. Broach, The role of phosphatases in TOR signaling in yeast, Curr. Top. Microbiol. Immunol. 279 (2004) 19–38.
[6] T. Beck, M.N. Hall, The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors, Nature 402 (1999) 689–692.
[7] M.E. Cardenas, N.S. Cutler, M.C. Lorenz, C.J. Di Como, J. Heitman, The TOR signaling cascade regulates gene expression in response to nutrients, Genes Dev. 13 (1999) 3271–3279.
[8] J.S. Hardwick, F.G. Kuruvilla, J.K. Tong, A.F. Shamji, S.L. Schreiber, Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 14866–14870.
[9] Y. Jiang, J.R. Broach, Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast, EMBO J. 18 (1999) 2782–2792.
[10] E. Jacinto, B. Guo, K.T. Arndt, T. Schmelzle, M.N. Hall, TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway, Mol. Cell 8 (2001) 1017–1026.
[11] R.T. Elder, M. Yu, M. Chen, X. Zhu, M. Yanagida, Y. Zhao, HIV-1 Vpr induces cell cycle G2 arrest in fission yeast (*Schizosaccharomyces pombe*) through a pathway involving regulatory and catalytic subunits of PP2A and acting on both Wee1 and Cdc25, Virology 287 (2001) 359–370.
[12] M. Masuda, Y. Nagai, N. Oshima, K. Tanaka, H. Murakami, H. Igarashi, H. Okayama, Genetic studies with the fission yeast *Schizosaccharomyces pombe* suggest involvement of wee1, ppa2, and rad24 in induction of cell cycle arrest by human immunodeficiency virus type 1 Vpr, J. Virol. 74 (2000) 2636–2646.
[13] Y. Zhao, J. Cao, M.R.G. O'Gorman, M. Yu, R. Yogev, Effect of human immunodeficiency virus Type 1 protein R (*vpr*) gene expression on basic cellular functions of fission yeast *Schizosaccharomyces pombe*, J. Virol. 70 (1996) 5821–5826.
[14] P. Fantes, Control of cell size and cycle time in *Schizosaccharomyces pombe*, J. Cell Biol. 24 (1977) 51–67.
[15] C. Alfa, P. Fantes, J. Hyams, M. McLeod, E. Warbrick, General Introduction to *S. pombe*, Experiments with Fission Yeast, A Laboratory Course Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1993, pp. 1–11.
[16] Y. Zhao, R.T. Elder, M. Chen, J. Cao, Fission yeast expression vectors adapted for large scale cloning and GFP fusion with positive screening, BioTechniques 25 (1998) 438–444.
[17] K. Maundrell, Thiamine-repressible expression vectors pREP and pRIP for fission yeast, Gene 123 (1993) 127–130.
[18] R. Fluri, J.R. Kinghorn, The all2 gene is required for the induction of the purine deamination pathway in *Schizosaccharomyces pombe*, J. Gen. Microbiol. 131 (Pt. 3) (1985) 527–532.
[19] S. Forsburg, Comparison of *Schizosaccharomyces pombe* expression systems, Nucleic Acids Res. 21 (1993) 2955–2956.
[20] R. Kaur, S.S. Ingavale, A.K. Bachhawat, PCR-mediated direct gene disruption in *Schizosaccharomyces pombe*, Nucleic Acids Res. 25 (1997) 1080–1081.
[21] D.S. McNabb, S.M. Pak, L. Guarente, Cassette for the generation of sequential gene disruptions in the yeast *Schizosaccharomyces pombe*, Biotechniques 22 (1997) 1134–1139.
[22] J.B. Keeney, J.D. Boeke, Efficient targeted integration at leu1-32 and ura4-294 in *Schizosaccharomyces pombe*, Genetics 136 (1994) 849–856.
[23] C. Alfa, P. Fantes, J. Hyams, M. McLeod, E. Warbrick, Nutritional control of entry into stationary phase determined by flow cytometry, Experiments with Fission Yeast, A Laboratory Course Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1993, pp. 52–56.
[24] K.E. Sawin, P. Nurse, Regulation of cell polarity by microtubules in fission yeast, J. Cell Biol. 142 (1998) 457–471.
[25] R.Y. Zhao, M. Bukrinsky, R.T. Elder, HIV-1 viral protein R (Vpr) and host cellular responses, Indian J. Med. Res. 121 (2005) 270–286.
[26] Y. Zhao, R.T. Elder, Yeast perspectives on HIV-1 VPR, Front. Biosci. 5 (2000) D905–D916.
[27] R. Rothstein, Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast, Methods Enzymol. 194 (1991) 281–301.
[28] R. Weisman, M. Choder, The fission yeast TOR homolog, tor1+, is required for the response to starvation and other stresses via a conserved serine, J. Biol. Chem. 276 (2001) 7027–7032.
[29] J. Marks, I.M. Hagan, J.S. Hyams, Growth polarity and cytokinesis in fission yeast: the role of the cytoskeleton, J. Cell Sci., Suppl. 5 (1986) 229–241.
[30] T. Matsusaka, D. Hirata, M. Yanagida, T. Toda, A novel protein kinase gene ssp1+ is required for alteration of growth polarity and actin localization in fission yeast, EMBO J. 14 (1995) 3325–3338.

[31] J.M. Mitchison, P. Nurse, Growth in cell length in the fission yeast *Schizosaccharomyces pombe*, J. Cell Sci. 75 (1985) 357–376.
[32] N. Kinoshita, H. Ohkura, M. Yanagida, Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle, Cell 63 (1990) 405–415.
[33] N. Kinoshita, H. Yamano, H. Niwa, T. Yoshida, M. Yanagida, Negative regulation of mitosis by the fission yeast protein phosphatase ppa2, Genes Dev. 7 (1993) 1059–1071.
[34] M. Kawai, A. Nakashima, M. Ueno, T. Ushimaru, K. Aiba, H. Doi, M. Uritani, Fission yeast tor1 functions in response to various stresses including nitrogen starvation, high osmolarity, and high temperature, Curr. Genet. 39 (2001) 166–174.
[35] L. Taricani, H.E. Feilotter, C. Weaver, P.G. Young, Expression of hsp16 in response to nucleotide depletion is regulated via the *spc1* MAPK pathway in *Schizosaccharomyces pombe*, Nucleic Acids Res. 29 (2001) 3030–3040.